IDENTIFICATION AND CHARACTERIZATION OF A SERPIN FROM *EIMERIA ACERVULINA*

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ABSTRACT: Serpins are serine protease inhibitors that are widely distributed in metazoans, but they have not been characterized previously in *Eimeria* spp. A serpin from *Eimeria acervulina* was cloned, expressed, and characterized. Random screening of an *E. acervulina* sporozoite cDNA library identified a single clone (D14) whose coding region shared high similarity to consensus structure of serpins. Clone D14 contained an entire open reading frame consisting of 1,245 nucleotides that encode a peptide of 413 amino acids, with a predicted molecular weight of 45.5 kDa and containing a signal peptide 28 residues. By Western blot analysis, polyclonal antiserum to the recombinant serpin (rbSp) recognized a major 55-kDa protein band in unsporulated oocysts and in oocysts sporulated up to 24 hr (fully sporulated). The anti-rbSp detected bands of 55 and 48 kDa in sporozoites (SZ) and merozoites (MZ), respectively. Analysis of MZ secretion products revealed a single protein of 48 kDa that may correspond to secreted serpin. By immunostaining, the serpin was located in granules distributed throughout both the SZ and MZ, but granules seemed to be concentrated in the parasites' anterior. Analysis of the structure predicts that the *E. acervulina* serpin should be an active inhibitor. However, rbSp was without inhibitory activity against common serine proteases. By Western blot analysis, the endogenous serpin in MZ extracts did not form the expected high-molecular-weight complex when coincubated with either trypsin or subtilisin. The results demonstrate that *E. acervulina* contains a serpin gene and expresses a protein with structural properties similar to an active serine protease inhibitor. Although the function of the *E. acervulina* serpin remains unknown, the results further suggest that serpin is secreted by the parasite where it may be involved in cell invasion and other basic developmental processes.

Although both coccidostats and live vaccines are widely used as control measures, coccidosis caused by several species of *Eimeria* remains an economically important disease of poultry. Reduced weight gain, and decreased efficiency of feed conversion associated with the disease, results in significant economic loss to the poultry industry (Williams, 1998; Allen and Fetterer, 2002). The development of drug resistance that has been reported for many years continues to reduce the efficacy of common anticoccidials (Chapman, 1993; Stephen et al., 1997; Williams, 2006). Available vaccines, although effective, are costly, and they require the use of live parasites; due to antigenic strain variation, vaccine efficacy can differ geographically (McDonald et al., 1988; Fitz-Coy, 1992; Innes and Vermeulen, 2006; Morris et al., 2007). Although several recombinant vaccine candidate proteins have been proposed, effective recombinant subunit vaccines have not yet been developed (Jenkins, 1998; Vermeulen et al., 2001; Innes and Vermeulen, 2006).

The search for targets for development of new controls requires a detailed understanding of genes and proteins and their expression during development of *Eimeria* species. The quest to characterize genes has been greatly enhanced by the production of an 8.4-fold shotgun sequence of the approximately 55 megabase pairs of *E. tenella* genome (Shirley et al., 2004; http://www.Sanger.ac.uk/Projects/E_tenella). In addition, efforts to characterize expressed sequence tags (ESTs) from various stages of *E. tenella* (Wan et al., 1999; Ng et al., 2002, Miska et al., 2004; Klotz et al., 2005) and more recently *E. acervulina* (Miska et al., 2008) have identified genes that may be important in understanding development of *Eimeria* spp.

Examinations of ESTs from *E. tenella* (Ng et al., 2002; Miska et al., 2004) have identified genes with strong homology to serpin, although serpin was less than 2% of the total ESTs examined (Ng et al., 2002). Serpins are part of a large superfamily of proteins, usually consisting of 350–400 amino acids, which often function as inhibitors of serine proteases and, by regulating proteolytic cascades, are essential to many physiological

processes (Huber and Carrell, 1989; Irving et al., 2000; Roberts et al., 2004). However, there are members of the serpin family, such as chicken ovalbumin, thyroxin-binding globulin, and cortisol-binding globulin, that do not inhibit proteases (Silverman et al., 2001). Maspin, which blocks the motility of tumor cells, is included in this group of serpins (Sheng et al., 1996).

A gene encoding a 43-kDa serpin protein has been reported from the parasitic protozoan *Entamoeba histolytica*. The recombinant serpin was not able to inhibit a panel of serine proteases, but the native 48-kDa protein secreted by the parasite interacted with cathepsin G produced by lymphocytes in vitro (Riahi et al., 2004).

Inhibitors of the serpin family have not been characterized in Apicomplexa, although protease inhibitors have been reported. Multidomain Kazal type serine protease inhibitors (TgPI-1 and TgPI-2) were identified in *Toxoplasma gondii* (Morris et al., 2002; Morris and Caruthers, 2003). TgPI-1, which inhibits many serine proteases, is secreted from dense granules into the parasitophorous vacuole upon invasion of the host cell, and it may play a role in regulation of invasion events. A 79-amino acid inhibitor with selectivity for inhibition of subtilisin, which may correspond to one domain of a Kazal type inhibitor, was identified in *Neospora caninum* (Bruno et al., 2004).

Serpins have not been characterized previously from *Eimeria* species. In the present study, we present the cloning, sequencing, and partial characterization of a serpin from *E. acervulina*.

MATERIALS AND METHODS

Host and parasites

Chickens (80–100 sex-sals, Moyers Hatcheries Inc., Quakertown, Pennsylvania), 4–5 wk of age, were infected with 1.0×10^5 *E. acervulina* (strain 12) oocysts per bird, placed in feed. On days 6–7 postimmunization (PI), unsporulated oocysts were collected from feces of *E. acervulina*-infected birds. For studies of sporulation time course, unsporulated oocysts were suspended in phosphate-buffered saline (PBS) containing an antibiotic/antimycotic mixture (Invitrogen, Carlsbad, California) and incubated under aeration at 29 C. At the desired time interval (ranging from 0 to 24 hr), an aliquot containing about 1×10^8 oocysts was removed from the incubation flask, centrifuged, and the pellet containing oocysts was resuspended in 1.0 ml of 40 mM Tris and stored at −70 C.

Sporozoites (SZ) were prepared from fully sporulated oocysts as described previously (Fetterer and Barfield, 2003), and merozoites (MZ) were collected at 89 hr PI from the duodenum from birds inoculated with 1×10^5 sporulated oocysts per bird and isolated as described previously (Fetterer et al., 2007). Isolated SZ and MZ were resuspended in 40 mM Tris and frozen at -70 C.

Cloning and expression of serpin gene

cDNA library construction: Total RNA was isolated from frozen *E. acervulina* sporozoites using TRIzol (Invitrogen) by following manufacturer's recommended protocol. Total RNA was resuspended in 100 µl of diethyl pyrocarbonate-treated water. The integrity of the RNA was determined by electrophoresing 1.3 µg of denatured total RNA on 1% agarose gel. Approximately 1.3 µg of total RNA was used as template for cDNA synthesis. Double stranded cDNA was generated using the SuperScript Choice System for cDNA synthesis (Invitrogen) and oligo(dT) primers. The ends of the double stranded cDNA were blunted by incubating for 45 min at 37 C with 20 U of DNA Ligase and 8 U of T4 DNA polymerase (Hoffman-La Roche, Nutley, New Jersey). The cDNA was ligated for 1 hr at room temperature into pBluescript II KS+ vector (Stratagene, La Jolla, California) using T4 DNA ligase (Hoffman-La Roche). Vector DNA was digested with EcoRV (New England Biolabs, Ipswich, Massachusetts) and dephosphorylated with alkaline phosphatase (New England Biolabs) before ligation. Ligated cDNA was transformed into TOP10 *Escherichia coli* (Invitrogen). Random clones were picked and grown in Luria-Bertani (LB) broth/ampicillin overnight and were subsequently frozen in glycerol at -70 C in 384-well plates. Clones were picked from glycerol stocks and grown overnight in LB broth/ampicillin. Plasmid DNA was isolated using QIAprep Miniprep kit (QIAGEN, Valencia, California).

Sequencing: All sequencing reactions were performed in this study using the Big Dye 3.2 sequencing kits (Applied Biosystems, Foster City, California) with nonisotopic dye terminators and analyzed on an automated sequencer (3730xl DNA sequencer, Applied Biosystems). Sequences obtained were compared with those in GenBank using the BLASTN or BLASTX algorithms (Altschul et al., 1990). Chromatograms were viewed and edited using the Sequencher 4.2 program (Gene Codes Corp., Ann Arbor, Michigan). Nucleotide sequence data reported in this study is available in the GenBank™, European Molecular Biology Laboratory, and DNA Data Bank of Japan databases under the accession number EU348753. The alignment was made using ClustalX software (Thompson et al., 1997), and the formatting was done using MacBoxShade 2.15E.

Construction of recombinant E. acervulina serpin: The entire coding sequence of *E. acervulina* serpin was polymerase chain reaction (PCR)-amplified from clone D14 using primers 132 (5′-GGC**GGATCC**ATG GCATTATTAAGTAA-3′) and 133 (5′-AT**GAGCTC**TTACTGCTGT GCAGCTGTCGG-3′), thus incorporating BamHI and SacI restriction sites into the resulting products. Products were digested with both BamHI and SacI (New England Biolabs), gel-purified using spin columns (QIAGEN), ligated into BamHI–SacI-digested pET28(a) vector (EMD Biosciences, San Diego, California) and pQE30 vector (QIAGEN), and transformed into TOP10 cells (Invitrogen). The maintenance of the correct reading frame was confirmed by sequencing positive transformants in both directions with T7 promoter (5′-TAATACGACT CACTATAGGG-3′) and T7 terminator (5′-GCTAGTTATTGCTCAG CGG-3′) primers (EMD Biosciences). Positive clones were then transformed into Rosetta 2 competent cells (EMD Biosciences). Bacteria containing the recombinant *E. acervulina* serpin were cultured in 50 ml of LB broth containing 50 µg/ml kanamycin until reaching an optical density$_{600}$ of 0.5. Expression was induced by adding isopropyl-1-thiogalactopyranoside (Sigma, St. Louis, Missouri) to a final concentration of 1 mM for 4 hr at 30 C. Cells were pelleted, resuspended in native binding buffer (1.2 mM NaH_2PO_4, 18.9 mM $Na_2H_2PO_4$, and 0.5 M NaCl, pH 7.8) containing 0.5 mM phenyl methanesulfonyl fluoride (Sigma), and frozen at -70 C. Cells were subjected to 3 freeze-thaw cycles between a dry ice-ethanol bath and 37 C water bath. RNase and DNase (1 µg/ml) were added, and the samples were rocked for 30 min at room temperature. Samples were then centrifuged at 7,000 rpm for 20 min at 4 C. The insoluble pellet was suspended in denaturing binding buffer (native binding buffer + 8 M urea) and extracted for 30 min on a rocker at room temperature. The cell extract was centrifuged at 7,000 rpm for 20 min at 4 C. The resulting supernatant was loaded onto a 1-ml nickel-nitrilotriacetic acid column. The column was washed 5 times with native binding buffer, followed by 5 washes with native wash buffer (12.3 mM NaH_2PO_4, 7.8 mM $Na_2H_2PO_4$, 0.5 M NaCl, pH 6.3, and 8 M urea). Recombinant protein was eluted 5 times with 1 ml of elution buffer (20 mM NaH_2PO_4, 0.5 M NaCl, pH 4.0, and 8 M urea). Recombinant protein was electrophoresed on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, and visualized by staining with Coomassie Blue (Sigma). *Escherichia coli* Rosetta cells harboring nonrecombinant pET28(a) or pQE30 vectors were used as negative controls. For use in protease inhibition assays, recombinant serpin (expressed in pQE30 vector) in elution buffer (3 ml) was made 5 mM with dithiothreitol (DDT) and dialyzed versus 20 mM Na_2PO_4 and 5 mM DTT, pH 7.5, for 3 hr at room temperature and then overnight at 4 C. The protein was then dialyzed overnight versus 20 mM Na_2HPO_4. Samples were concentrated to about 0.5 ml and stored at 4 C before use in assays. Protein concentration was estimated from predicted extinction coefficient.

Antibody production

Female New Zealand White rabbits, 9–11 kg and specific pathogen-free (Charles River Laboratories, Inc., Wilmington, Massachusetts), were bled via the central auricular artery, before immunization to obtain baseline control sera. Rabbits were immunized using the ImmuMax SR adjuvant system (Repros Therapeutics, Inc., The Woodlands, Texas; Fetterer and Barfield, 2003) and 200 µg of recombinant serpin (expressed in pET28a vector. After 30 days, rabbits were immunized again with 200 µg of recombinant serpin. At about 37 days PI, a second blood sample was obtained to determine positive antibody response and titer.

Protein extracts

Oocysts (1×10^8) suspended in 1 ml of lysis buffer (1.0 % Triton X-100, 3 mM $MgCl_2$, 10 mM NaCl, and 10 mM Tris, pH 8.0) were placed in a 1.5-ml capped microfuge tube containing 0.5 g of 0.5-mm glass beads and homogenized with a mini BeadBeater (BioSpec Products, Bartlesville, Oklahoma). Soluble extracts were prepared as described previously (Fetterer and Barfield, 2003). SZ and MZ ($1 \times 10^8 - 1 \times 10^9$) parasites per milliliter were sonicated in 1.0 ml of LB broth for 3 cycles (5 min each) on ice. Parasite homogenates were stored frozen at -70 C.

Eimeria acervulina MZ (1×10^9) were incubated in 1 ml of culture media (CM; modified Eagle's medium containing 2.5% fetal bovine serum). Samples were incubated for 2 hr at 41 C under an atmosphere of 95% air-5% CO_2. After centrifugation (10,000 g for 10 min at 4 C), the supernatant was removed and frozen at -70 C. The MZ were washed once with PBS and frozen. After incubation, parasites were centrifuged, and the supernatant was removed and frozen at -70 C. The pellets containing intact MZ were washed with PBS and frozen at -70 C. Concentrations of soluble proteins were measured with bicinchoninic acid assay (Pierce Chemical, Rockford, Illinois), with bovine serum albumin as the standard.

Electrophoresis

Protein samples were analyzed by polyacrylamide gel electrophoresis using 1-mm-thick gradient gels (8 × 9 cm, 4–12% Bis Tris, Invitrogen) as described previously (Fetterer and Barfield, 2003). Western blot analysis was performed using the basic method described previously (Fetterer and Barfield, 2003), except that the primary antibody consisted of a 1:500 or 1:1,000 dilution of rabbit polyclonal antibody to *E. acervulina* prepared as described above.

Protease inhibitor assays

Inhibitory activity against purified proteases: Inhibitory activities of rbSp and a MZ extract were determined by incubation with one of several purified serine proteases. The assay consisted of 170 µl of reaction buffer (RB; 50 mM Tris, and 150 mM NaCl, pH 7.8; for subtilisin assay, the RB also contained 10 mM $CaCl_2$ and 0.005% Triton X-100), 20 µl of protease, 10 µl of rbSp (200 nm) or MZ extract (25 µg protein), and 10 µl of substrate (0.1-1.0 mM). Controls contained 10 µl of RB in place of inhibitor. Inhibitor and protease were preincubated for 15 min before addition of substrate. The samples were incubated at 37 C, and absorbance at 405 nm was measured with a spectrophotometer. The

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                        . . . .10 . . . .20 . . . .30 . . . .40 . . . ↓.50 . . . .60 . . . .70 . . . .80 . . . .90 . . . 100
E. ACERVULINA      ..................MALLSKLTRFCSAAAVATALSLSSTASAMDPSTVTAERLYGEIAGPSLEAK..PNFVFSPFSIFSVFHTAQKGAAGETRGQM
E. TENELLA         ...................-------KGVYL-F-I-G-FPFT-MT---ER--I-------K---A-IA--..------I--------A-------Q-KA--
HUMAN ANTITRYPSIN  MPSSVSWGILLLAGLCCLVPVSLAEDPQGD--QKTDTSHHDQDHPTFNKI-PNLAEFAFSYRQLAHQSNS.T-IF---V--ATA-AMLSL-TKAD-HDEI
CHICKEN OVALBUMIN  .............................................-GSIGAASMEFCFDVFKELKVHANE--IFYC-IA-M-ALAMVYL--KDS--T-I
E. HISTOLYTICA     ....................................M-MDYQDIENMQIALYK-CVDWYN.-SPI-..EDI---TH-M-IA-SLLYI---A--KT-L
C. REINHARDTII     ........................MPP-KAPVADAPASP-LSALSKASYA-FL-A-AGTGGK....GCFL--M--IYALTL-LN--GPK-STHS
G. LAMBLIA         .......................MATALPQALFSD-VAYFTEERLQS..KGV-Y-SW-L-HTLFIFLAAVT-SVHDDL

                        . . . 110 . . . 120. . . 130 . . . 140 . . . 150 . . . 160 . . . 170 . . . 180 . . . 190 . . . 200
E. ACERVULINA      DALVGPNES...................FAIPELIQPPQKEGAVAVDVANRLYVHPGLEKNKHFKKFKKQLQEEKQSAETIDFSDGAAAAEKINSFVAT
E. TENELLA         -------T.................-E--------R-D---T------------------N--Q-N------ED--HE---------------A----
HUMAN ANTITRYPSIN  LEGLNF-LTEIP..........EAQIHEGFQEL-RTLN-PDSQLQLTTG-G-FLSE--KLVDK-LEDV-K-Y..HSE-F-VN-G-TEE-KKQ--DY-EK
CHICKEN OVALBUMIN  NKV-RFDKLPGFGDSIEAQCGTSVNVHSSLRDI-N-ITKPNDVYSFSL-S---AEERYPILPEYLQCV-E-YRGGLEPINFQTAAD.Q-R-L---W-ES
E. HISTOLYTICA     EKVF-FASIPE.............RNFIKFLQSI-KQQDPTMS-T--IV-GIWASQK--FTEEY---AITT-DCQLKNVNFGND-E..NIRQE--K--EE
C. REINHARDTII     EL-AAIAGGAENAA......NLGEGDLNSELGRTMTLLNGA--G-AEGSASRM-LANSVWTHRGTTLR-EYVDAM--LFDATAREAVNG-KDV-AW-EG
G. LAMBLIA         LTAMQLS-K.......................EAS--AVSQIASLLREDSALALAANIYA-SVSPTVEFVALA-R..YFDITIEPLKS-AQV-NWCSA

                        . . . 210 . . . 220 . . . 230 . . . 240 . . . 250 . . . 260 . . . 270 . . . 280 . . . 290 . . . 300
E. ACERVULINA      TTRDHIKNLLNASALGPQTRLVLINALYFKAPWLQQFESSATSLGVFFT.PSGPKQTQFMKGKLDKAPLLLSMKKE..ALAVGLPYTDPRLRLYIVMPED
E. TENELLA         --I---I----VSP----A-----V---------T--SAAG--K---S-.-A----VM--R---------VT-----.F-----I--S-A-----FF--D-
HUMAN ANTITRYPSIN  G-QGK-VD-VKELD..RD-VFA-V-YIF--GK-ERP--VKD-EEED-HVDQVTTVKVPM--RLGMFNIQHCKKLSS...WVLLMK-LGN.ATAIFFL-DE
CHICKEN OVALBUMIN  Q-NGI-R-V-QP-SVDS--AM--V--IV--GL-EKA-KDED-QAMP-RVTEQES-PV-M-YQIGLFRVASMASE-MK...ILE--FASGTMSMLVLL-DE
E. HISTOLYTICA     A--KV-VDF-QPGTISGD-IA-IV--I---GE-ETP-KIVQKKMKFE.....-DEEVV--ERIECS...AVFTEK..YTS-SI--VGNQYSMV-I--NN
C. REINHARDTII     V-KGM--D-VQT....D-WDAI-A--I---GF-THA-KKED-FP-E-T-GAKAT-EVP--CKNFEAQDRITAVR--GLYD--A---KGDAFSAIALL-AV
G. LAMBLIA         Q-KGL-NSIVDD...VKD-DAI--S-I----N-ALK-SKDD-KMLP-TSLDGRTQDREL-YL-AKLQYASSDSAQM.....-K-N-N-SNVSAI--L-KE

                        . . . 310 . . . 320 . . . 330 . . . 340 . . . 350 . . . 360 . . . 370 . . . 380 . . . 390 . . . 400
E. ACERVULINA      LSSFEKSMIDNPQQLEQTFADMESSAMDRSFEEEIFLTLPKFKLSADDNKVDLREIFGRLGAPDMFDMGKADFSGITGDRDLFVSSFVHQADIDVNEEGT
E. TENELLA         -A-----A-V-D--LI-TAIS------N--------MY--V-------------T----SM--T--RVD-----EM-----------------------
HUMAN ANTITRYPSIN  GKLQHLENELTHDIITK........FLENEDRRSAS-H---LSITGT...Y--KSVL-Q--ITKV-SN-.--L--V-EEAP-KL-KA--K-VLTID-K--
CHICKEN OVALBUMIN  V-GL-QLESIINFEKLT.....WTSSNVME-RK-KVY--RM-MEEK..YN-TSVLMAM-IT-V-SSS.-NL---SSAES-KI-QA--A-HAEI--A-R
E. HISTOLYTICA     MKE---ENMGELKEYVR........RTIQE-S-KRNV-I----IETS...FSMNQQLKQ--LINA--ER.....KMAKG.HFC--EAI-K-VVE-D-K--
C. REINHARDTII     GVAM-AALK-WGS..........GPQELRPVGKC-VK-----VESQ...LS-KTVLHK--VKQA-GGS.----RLSDT.KM--DV--K-VVE-D----
G. LAMBLIA         TGKASLKAALRYDN.........FFPT-GFLTK-VV-K----R-ESS...H--KDHLVLR--KS--GVCDCNG-...LGAPMS-TKVIQK-V-E-D----

                        . . . 430 . . . 440 . . . 450 . . . 460 . . . 470 . . . 480
E. ACERVULINA      EATAATAMIMMLRAMPM PKT.QINVLVDKPFIFQLRFVDGDTNLILFSGRVADPTAAQQ
E. TENELLA         -----S----------- ----P---II---V------------T----I---A----
HUMAN ANTITRYPSIN  --AG-....-F-E-I-- SIP..PE-KFN---V-LMIE..QN-KSP--M-K-VN--QK..
CHICKEN OVALBUMIN  -VVGSAEAGVDAASVSE EFR......A-H--L-CIKH..IA--AV--F--CVS-.....
E. HISTOLYTICA     I-A---GIAL-RCCL-L EP..PRD-IIN--YF-VI...I-EEQYP--F-K-SH-RFK..
C. REINHARDTII     V-A-----VM-LRC-L- T...PEFIFNR--A-IIMH..TP-G-PA-V-T-N--SQ...
G. LAMBLIA         T-A-V--VR-LRC--MA -PPEIYH-IC-R--W-M-ER....DGMP--LTALL.....
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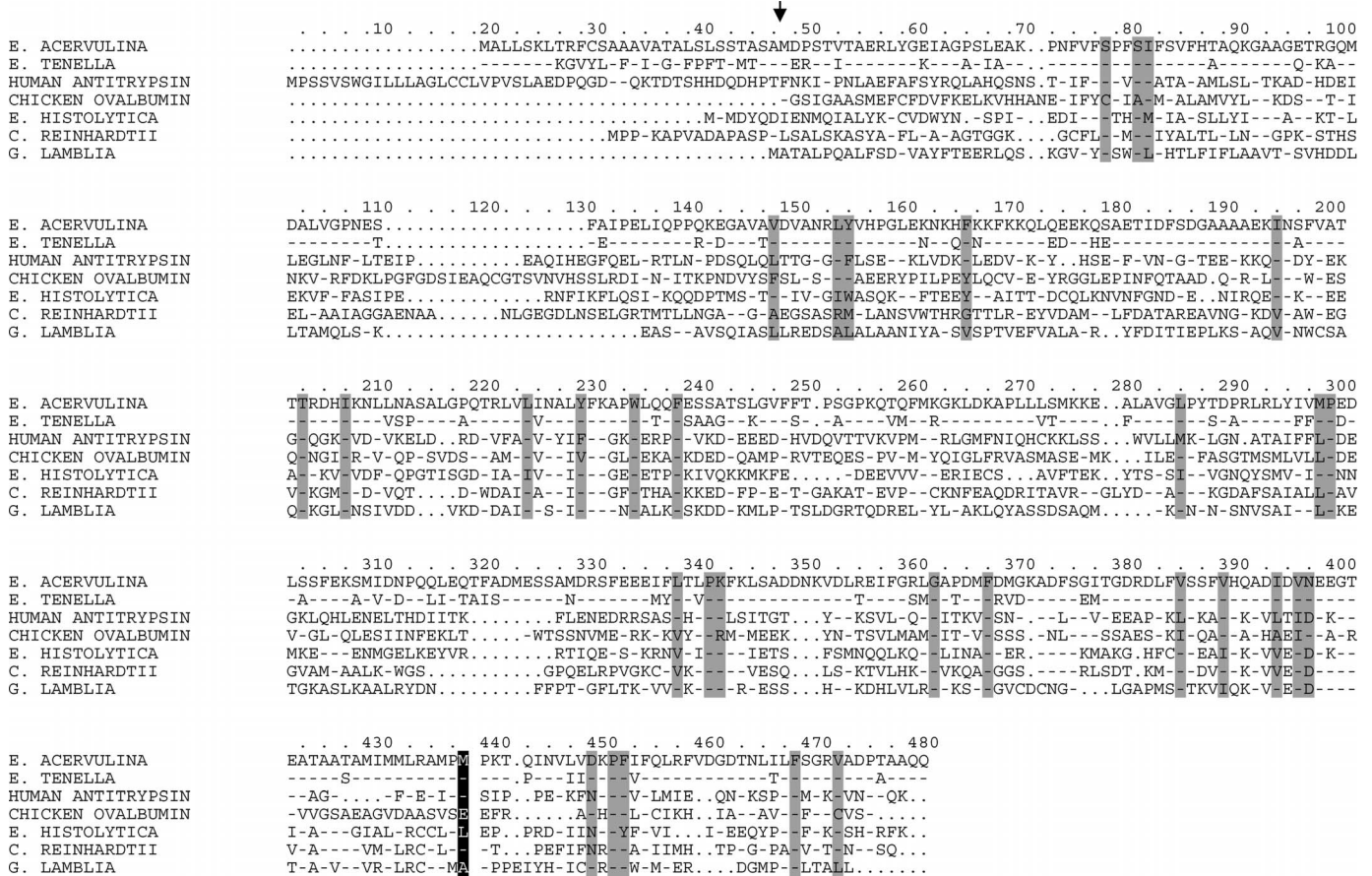
FIG. 1. Alignment of *E. acervulina* and *E. tenella* serpins with human antitrypsin, ovalbumin and putative serpins from *E. histolytica*, *Chlamydomonas reinhardtii*, and *Girardia lamblia*. The dashes denote sequence identity, and the dots denote sequence gaps. The 32 amino acids conserved in active serpins are shaded in gray. The P1 site is highlighted in black. The RCL spans between residue 397 and 432. The cleavage site for the leader sequence is indicated by the arrow.

enzymes and their *p*-nitroanalide (PNA) substrates used in the assays were bovine pancreatic trypsin (200 nM; Sigma) and Z-Arg-PNA (Bachem Biosciences, King of Prussia, Pennsylvania); bovine pancreatic chymotrypsin (10 nM; Sigma) and *N*-succinyl-Ala-Ala-Pro-Phe-PNA (Sigma); subtilisin A (100 nm; Sigma) and Z-Gly-Gly-Leu-PNA (Sigma), and porcine pancreatic elastase (100 nM; Sigma) and *N*-succinyl-Ala-Ala-Pro-Leu-PNA (Sigma).

Interaction of endogenous serpin with trypsin and subtilisin: Extracts of *E. acervulina* MZ (10 μg in 2 μl of RB) were mixed incubated with trypsin (1 or 10 ng) or subtilisin (0.01, 0.1, 1, or 10 ng) in a 12-μl total volume. Control incubations contained 2 μl of MZ extract plus 10 μl of RB. After incubation for 15 min at 22 C, 12 μl of 2× sample buffer was added to each sample and heated for 10 min at 70 C. Samples were analyzed by Western blot as described above.

Antibody staining

For indirect fluorescent antibody staining, SZ and MZ were washed with PBS and placed on slides. Parasites were either air-dried or fixed with or without methanol, and processed as described previously (Fetterer et al., 2004). Parasites were visualized at ×400 magnification with a compound microscope (Carl Zeiss Inc., Thornwood, New York).

RESULTS

Characterization of *Eimeria* serpin gene

Random screening of an *E. acervulina* sporozoite cDNA library identified a single clone (D14) whose coding region shared high similarity (E value of 7e−49) to consensus struc-ture of single domain serpins. Clone D14 contained an entire open reading frame consisting of 1,245 nucleotides that encodes a peptide of 413 amino acids. The clone also included 104 base pairs (bp) of the 5′ untranslated region (UTR) and 194 bp of the 3′ UTR; however, a polyadenylation signal could not be located. By carrying out a BLAST search, the *E. acervulina* serpin sequence was shown to be highly similar to an unpublished serpin sequence from a closely related species, *Eimeria tenella* (AJ920258). Analysis of the *E. acervulina* serpin using the SignalP version 3.0 software (Emanuelsson et al., 2007) indicated that the protein most likely contains a signal peptide of 28 amino acids, with a hypothetical cleavage site located between alanine and methionine (indicated as an arrowhead shown in Fig. 1). The 2 serpins from these coccidians are shown aligned in Figure 1 with human antitrypsin, ovalbumin, and 3 putative serpins from other single-cell eukaryotic organisms (*E. histolytica*, *Chlamydomonas reinhardtii*, and *Giardia lamblia*). The amino acid identity between *E. acervulina* and *E. tenella* serpins is 77.7%. There are 32 amino acids that are 100% conserved in active serpins, which are shaded in gray (Fig. 1). All but 2 of these are conserved between *Eimeria* spp. serpins and human antitrypsin. The nonconserved residues are substitutions at position 396 from an aspartic acid in the antitrypsin sequence to asparagine in the *Eimeria* spp. serpin sequences. The second
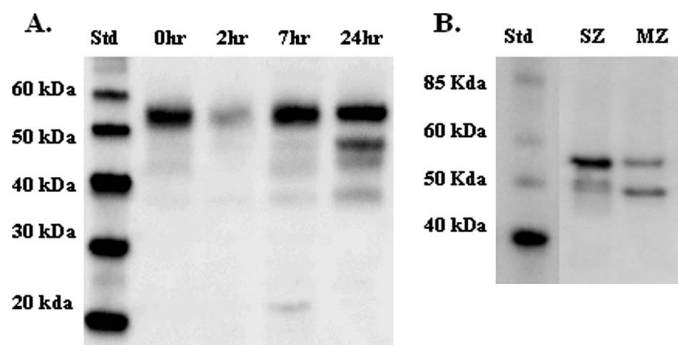
FIG. 2.   Western blot analysis of extracts from developmental stages of *E. acervulina* and *E. tenella* probed with anti-rbSp. (**A**) *Eimeria acervulina* oocysts from 0 to 24 hr of sporulation and (**B**) extracts of SZ and MZ. For oocyst extracts, 10 μg of protein per lane (equivalent to about 1×10^6 oocysts) was applied to each lane. The equivalent of 1×10^6 parasites per lane was used for extracts of SZ or MZ. Anti-serpin was used at a dilution of 1:500.
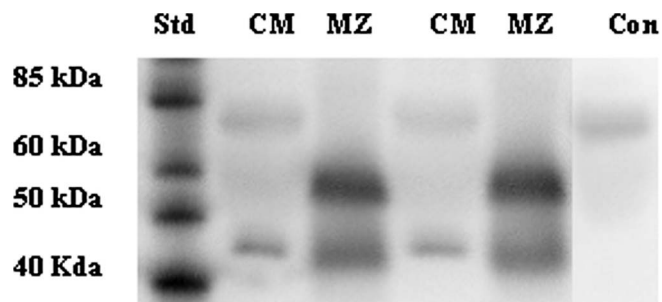

FIG. 3.   Western blot analysis with anti-rbSp of CM and extracts of *E. acervulina* MZ and controls containing CM only (Con) after 2-hr incubation in CM. Duplicate incubations are shown. Lanes contained 10 μl of CM or 10 μl of a 1-ml extract of MZ (equivalent to 1×10^7 MZ). Anti-rbSp was used at a dilution of 1:500.

substitution involves an asparagine in position 448 in human antitrypsin and an aspartic acid in *Eimeria* spp. serpin sequences. The P1 site is highlighted in black in Figure 1 and is conserved among all sequences shown except for chicken ovalbumin. The reactive center loop (RCL) of *Eimeria* spp. serpins hypothetically spans between residues 397 and 437. It is apparent that the RCL of unicellular eukaryotic organisms is longer than that of human antitrypsin.

Developmental expression of serpin

Antibody to rbSp detected a major protein band of 55 kDa in extracts of *E. acervulina* oocysts from 0 hr (unsporulated) to 24 hr of sporulation (fully sporulated), with some minor bands of lower molecular weight also present (Fig. 2A). In addition to the 55-kDa band, a relatively abundant band of 48 kDa was also detected in oocysts after 24 hr of sporulation. The anti-rbSp detected bands of 55 and 48 kDa in SZ extracts and 55 and 48 kDa in MZ extracts (Fig. 2B).

Secretion of serpin by *E acervulina* MZ

Western blot analysis with anti-rbSp identified a 48-kDa band in CM after incubation with 1×10^9 MZ for 2 hr at 41 C, whereas 55- and 48-kDa bands were observed in extracts of MZ (Fig. 3). By densitometry, the 48-kDa band in the CM represented about 30% of the equivalent band in MZ extract. A minor band of 73 kDa was present in both CM with and without MZ and represents an artifact contained in CM.

Protease inhibition assay

The rbSp was without detectable activity on the activity of trypsin, chymotrypsin, porcine elastase, and subtilisin. Extracts of *E. acervulina* MZ equivalent to 1×10^7 MZ did not inhibit the proteolytic activity of trypsin, but about 18% decrease in porcine elastase and 29% decrease subtilisin activity were noted (data not shown).

Incubation of MZ extract with trypsin or subtilisin did not result in formation of higher molecular weight complexes that might be expected if a covalent interaction occurs between the protease and serpin (Fig. 4). Control incubations probed with

the anti-rbSp identified a predominant band of 55 kDa, with a minor band of 48 kDa. When incubated with 10 ng of trypsin, the protein recognized by anit-rbSp (55 kDa) was greatly reduced, with a band of 48 kDa present with small amounts of bands of lower molecular weights. When incubated with 1 ng of trypsin, the 55-kDa band was largely intact, with small amount a 48-kDa band visible. Subtilisin (10 or 1 ng) eliminated the 55-kDa band. Incubation with 0.1 or 0.01 ng of subtilisin yielded a 48-kDa band as well as several lower molecular weight bands.

Antibody staining

Antibody staining with anti-rbSp localized serpin to granular structures within both SZ (Fig. 5A) and MZ (Fig. 5B). MZ stained with serum from rabbit before immunization with rbSp were unreactive (Fig 5C). The patterns of staining for SZ and MZ were similar. In general, granules staining with anti-rbSp were more concentrated in the parasite's anterior. Anti-rbSp also stained schizonts that copurified with MZ (Fig. 5B).

DISCUSSION

Although serpins represent a large superfamily of proteinase inhibitors that are widely distributed in metazoans, the current result is the first characterization of a serpin in a member of the Apicomplexa. In addition to sequence homology, the *E. acervulina* serpin has structural features that suggest it is most likely an active serine protease inhibitor. The *E. acervulina* serpin contains sequences near its C terminus with strong homology to the RSL for active serpins (Irving et al., 2000; Silverman et al., 2001). The RSL that contains the proteinase recognition site is essential for the inhibitory function of serpins. In contrast to the predicted inhibitory activity, rbSp was inactive when test-
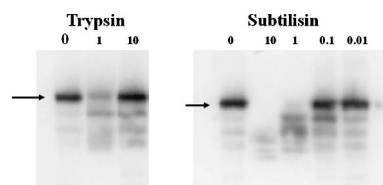

FIG. 4.   Western blot analysis with anti-rbSp after incubation of MZ extract with (10–0.01 ng) or without (0) trypsin or serpin. Arrow indicates positions of 55- or 48-kDa bands respectively.
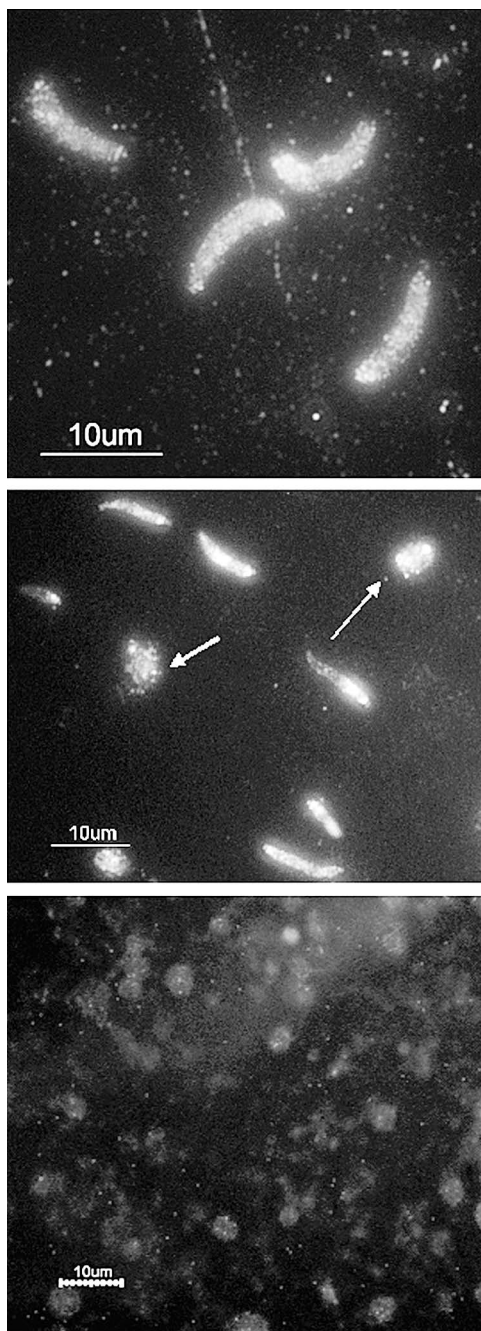
FIG. 5.   (**Top**) Staining of *E. acervulina* SZ. (**Middle**) MZ with anti-rbSb. (**Bottom**) MZ stained with preimmunization control sera. Arrows indicate schizonts that copurified with MZ.

ed against common potential serine protease targets. The reason for this is not clear. It is possible that the recombinant serpin lacks the appropriate native conformation essential for inhibitory activity or that essential cofactors are needed for complete activity of the serpin (Jordan et al., 1978; Wiman et al., 1988). It is also possible that none of the serine proteases tested is the target for the *E. aceruvulina* serpin. Similar to *E. acervulina*, a recombinant serpin from *E. histolytica* was inactive against a panel of serine proteases, but a secreted native serpin formed a complex with neutrophil cathepsin G (Riahi et al., 2004). In

addition to a lack of effect of rbSp on proteases, the endogenous serpin in MZ extracts did not form any stable higher molecular weight (about 75-kDa) complexes that might be expected if there was covalent binding of the serpin to the target protease. Instead, a digestion product of about 45–48 kDa was observed, particularly when lower concentrations of protease were used. The amount of digestion was dependent on protease concentration, and with higher concentrations of both trypsin and subtilisin, the endogenous serpin in MZ extracts was completely digested. This indicates that the endogenous serpin does not inhibit the serine proteases examined.

The RSL sequence of the *E. acervulina* serpin is very similar to the RSL of a serpin from the cephalochordate *Brachiostoma lanceolatum*, which has been recently shown to be an inhibitor of the cellular processing enzyme furin (Bentele et al., 2006). Although furins have not been reported from *Eimeria* spp., it is possible the furins or some other related serine protease are the target of the *Eimeria* spp. serpin. Interestingly, the *B. lanceolatum* serpin has an endoplasmic reticulum (ER) sequence, but an ER sequence is lacking in the *E. acervulina* serpin.

The *E. acervulina* serpin is expressed in unsporulated oocysts, during stages of sporulation and in SZ and MZ. Proteins associated with sporozoite function such as microneme proteins (Ryan et al., 2000) and a refractile body protein (Fetterer, Miska et al., 2007) show stage specific expression. They are absent during early stages of sporulation and are first detectable during formation of the SZ within the oocysts. Distribution of serpin in all the *E. acervulina* asexual stages examined indicates a more generalized function. However, the serpin seems to be localized to some extent in the anterior of the SZ and MZ as observed for microneme proteins, also suggesting a role in invasion. In other Apicomplexa, serine proteases have been implicated in microneme release and the invasion process so that colocalization of serine proteases and inhibitors may occur (Morris et al., 2002). Consistent with involvement of a serpin in invasion are the observations that serine protease inhibitors of the Kazal type have been implicated in invasion events in *T. gondii* (Morris et al., 2002); classical serine protease inhibitors reduce *E. tenella* SZ invasion in vitro (Fetterer, Jenkins et al., 2007).

There is evidence that *Eimeria* spp. serpin is secreted. Only a 48-kDa protein detected by the anti-rbSp antibody was present in the secretory products from *E. acervulina* MZ, whereas both a 48- and 55-kDa protein were present in MZ extracts, suggesting that the 48-kDa protein may be a secreted serpin. The 55-kDa protein is somewhat larger than predicted for the serpin (46 kDa); however, the 48-kDa is closer to the predicted molecular weight (43 kDa) of the serpin lacking the secretory leader sequence. The localization of the serpin to granules may also suggest a secretory function. Kazal type inhibitors characterized from *T. gondii* colocalize with dense granules that secrete a number of proteins upon invasion (Morris et al., 2003). However, dense granule protein homologs have not been reported from *Eimeria* spp., and the nature of the granules containing the serpin in *E. acervulina* is unknown.

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